HAEMATOLOGICAL CHANGES IN ADJUVANT DISEASE IN THE RAT

II. IRON METABOLISM AND ^{51}Cr ERYTHROCYTE SURVIVAL

BY

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It has been shown (Mikołajew, Kuratowska, Kossakowska, Płachecka, and Kopeć, 1969) that severe anaemia is a frequent finding in postadjuvant disease induced in rats by repeated injections of Freund's adjuvant (FA). The characteristic features of this anaemia were—hypochromicity, macrocytosis, high peripheral reticulocytosis, and erythroid hyperplasia of the bone marrow with the arrest of maturation at the stage of basophilic and polychromatophilic erythroblast.

In the present work, attempts to elucidate the pathogenic mechanisms of postadjuvant anaemia are presented. They include studies on iron metabolism and on erythrocyte survival.

Material and Methods

The rats were divided into four groups according to the number of FA injections as described previously (Mikołajew and others, in press). All examinations were performed during maximal exacerbation of joint involvement after subsequent FA injections.

The serum iron level was determined by the method of Schales (1958), serum iron-binding capacity by the method of Ramsay (1957), and concentration of non-haeme iron in organs by that of Heilmeyer and Plötnner (1937).

HCl washed glassware and deionized water were used. The livers and spleens were weighed and homogenized in a glass homogenizer and diluted with water to 5 per cent. weight/volume in the case of liver and to 2 per cent. in the case of spleen. For spectrophotometric determination a Unicam SP-500 spectrophotometer was used.

Incorporation of ^{59}Fe -citrate into Erythrocytes and Organs

Carrier-free 1 μC . ^{59}Fe -citrate (specific activity 5 $\mu\text{C}/\mu\text{g}$. Fe) in 1 ml. saline was injected intra-

peritoneally. The radioactivity of the blood and organ homogenates was determined after 24 hours. The percentage of the injected dose incorporated into erythrocytes was calculated from the formula:

$$\frac{\text{cpm/ml. of blood} \times \text{rat weight} \times 0.05 \times 100}{\text{cpm/ml. of standard}}$$

The standard ^{59}Fe solution contained 1 μC . ^{59}Fe per ml. Incorporation into organs was expressed as the percentage of the injected dose found in the whole organ or in 1 g. tissue.

^{59}Fe -labelled Haemoglobin and Incorporation of Iron from $^{59}\text{FeHb}$ into Erythrocytes and Organs

30 μC . ^{59}Fe -citrate was injected intraperitoneally into two rats, who were made anaemic by three injections of 2 ml. 2 per cent. phenylhydrazine solution given every second day. 72 hours after the ^{59}Fe injection, blood from the heart was withdrawn into heparin. Haemolysate was prepared by the method to Drabkin (1950) from twice-washed red cells. The combined haemolysate from two rats was used as ^{59}Fe -labelled haemoglobin. 1 ml. ^{59}Fe -Hb was injected intraperitoneally, and 24 hours later the rats were killed by cardiac bleeding under ether narcosis. The results were calculated in the same manner as ^{59}Fe -citrate incorporation.

Excretion of Iron in Faeces and Urine

The rats kept in metabolic cages were given 4 μC . ^{59}Fe -citrate intraperitoneally. Faeces and urine were collected separately for 31 days at intervals of 2 to 4 days.

Erythrocyte Survival

This was studied in ten control rats and in seven-
teen rats given a single injection of FA. 100 μC

$\text{Na}_2^{51}\text{CrO}_4$ (specific activity 100 $\mu\text{C./l } \mu\text{g.Cr}$) was injected intraperitoneally. 0.2 ml. blood from the heart was withdrawn 24 hours and 4 days after ^{51}Cr injection, and then twice a week for the next 28 days. Later the 0.5 ml. samples of blood were taken once a week. The two first samples were centrifuged, and radioactivity was measured in washed erythrocytes as well as in plasma combined with washing fluids. After checking that plasma was ^{51}Cr free on the fourth day, the radioactivity of the whole blood was counted. The results corrected for natural decay were expressed as percentages of extrapolated initial value.

Results

The changes in blood serum iron (BSI) during FA treatment are presented on Fig. 1. The mean value of BSI decreased after subsequent FA injections, being lowest in the rats given FA three times. The animals treated with two or three FA injections

^{59}Fe and ^{51}Cr were purchased from the Institute of Nuclear Research in Warsaw.

A well-type scintillation counter type EKCO N-550A was used for studies of the incorporation of ^{59}Fe into erythrocytes and organs, and a SE 2-well scintillation counter for survival of red blood cells and excretion studies.

were divided into two subgroups according to the Hb concentration. Hb values below 12.8 g./per cent. were considered to indicate anaemia; this degree of anaemia was observed in only two rats after a single FA injection and not at all in the control group. The BSI was found to be much lower in the animals with pronounced anaemia, but after the third injection of FA the BSI values in the rats without anaemia were much lower than in the control group and in rats after one FA injection, and they were nearly equal to the BSI values in anaemic rats after two FA injections. The total serum iron-binding capacity was observed to be the same in the controls and after two FA injections (Table I). The incorporation of ^{59}Fe injected intraperitoneally as ^{59}Fe -citrate into erythrocytes was found to increase after FA treatment.

TABLE I
TOTAL IRON-BINDING CAPACITY OF THE SERUM
($\mu\text{g. per cent.}$)

Controls		After Second FA Injection	
No. of Rats	Total Iron-Binding Capacity	No. of Rats	Total Iron-Binding Capacity
10	440 ± 61.53	10	449 ± 65.1

Fig. 1.—Serum Fe level after FA injections.

Fig. 2.—Incorporation of ⁵⁹Fe into erythrocytes 24 hours after intraperitoneal injection of ⁵⁹Fe-iron-citrate (per cent. of dose).

Fig. 2 shows that the percentage of radioactive iron found after 24 hours in peripheral red blood cells was higher after a single FA injection than in intact rats, and rose further after the second injection, particularly in the rats with pronounced anaemia. The same is true for incorporation of ⁵⁹Fe from labelled rat haemoglobin (Fig. 3). Its incorporation into erythrocytes was much higher than in controls in all rats which had two FA injections and was extremely high in anaemic rats.

Fig. 3.—Incorporation of ⁵⁹Fe from labelled haemoglobin into erythrocytes 24 hours after intraperitoneal injection of ⁵⁹Fe Hb. Total radioactivity of injected Hb was accepted as 100 per cent.

Studies on the iron in spleen and liver were preceded by examination of the weight changes in these organs (Table II). Spleen weight was found to increase after the first and third injections but to be unchanged after the second. The average liver weight was slightly smaller in all experimental groups as compared with the controls.

The results of examination of non-haeme iron in spleens and livers are presented in Tables III and IV. The iron concentration in the whole spleen increased significantly after the first injection, was equal to the control values after the second, and dropped to very low values after the third. On the other hand, when the results were calculated as Fe mg./g. tissue, the changes were not significant (Table III). The non-haeme iron in the liver expressed as mg. Fe in the whole organ was found to decrease after the second FA injection, and to become very low after the third (Table IV).

The accumulation of ⁵⁹Fe in the spleen and liver was studied 24 hours after ⁵⁹Fe-citrate (Tables V and VI) and ⁵⁹Fe-labelled rat haemoglobin intraperitoneal injections (Figs 4 and 5, overleaf).

After the first injection of FA the spleen accumulated a significantly higher percentage of the dose

TABLE II
CHANGES IN LIVER AND SPLEEN WEIGHT (g.)

No. of Rats	Controls	After FA Injections		
		First	Second	Third
	69	31	32	21
Spleen	0.478 ± 0.197	0.571 ± 0.204	0.47 ± 0.11	0.65 ± 0.215
Liver	8.7 ± 1.5	7.43 ± 3.3	7.65 ± 1.75	7.65 ± 1.3

TABLE III
NON-HAEME IRON IN THE SPLEEN OF CONTROL RATS AND AFTER FA INJECTIONS

No. of Rats	Controls	After First FA Injection	Controls	After Second FA Injection	Controls	After Third FA Injection
	27	30	12	24	11	23
Fe (mg. in spleen) ..	0.18 ± 0.05	0.23 ± 0.09	0.187 ± 0.03	0.19 ± 0.11	0.223 ± 0.05	0.064 ± 0.04
P		<0.01		>0.8		<0.001
Fe (mg./g. tissue) ..	0.37 ± 0.12	0.42 ± 0.16	0.404 ± 0.05	0.39 ± 0.17	0.392 ± 0.08	0.108 ± 0.07
P		>0.2		>0.7		<0.0001

Separate control groups were used because the control values varied depending on the duration of experiments.

TABLE IV
NON-HAEME IRON IN LIVER OF CONTROL RATS AND AFTER FA INJECTIONS

No. of Rats	Controls	After First FA Injection	Controls	After Second FA Injection	Controls	After Third FA Injection
	27	31	12	24	12	22
Fe (mg. in liver)	0.6 ± 0.151	0.57 ± 0.285	1.06 ± 0.152	0.79 ± 0.38	0.624 ± 0.22	0.305 ± 0.205
P		>0.04		<0.01		<0.001
Fe (mg./g. tissue) ..	0.069 ± 0.02	0.073 ± 0.02	0.103 ± 0.01	0.11 ± 0.03	0.094 ± 0.05	0.046 ± 0.03
P		>0.2		>0.1		<0.01

Separate control groups were used because the control values varied depending on the duration of experiments.

TABLE V
⁵⁵Fe IN SPLEEN 24 HOURS AFTER INTRAPERITONEAL INJECTION OF ⁵⁵Fe IRON-CITRATE
(Results in per cent. of injected doses)

No. of Rats	Controls	After First FA Injection (Whole Group)	Controls	After Second FA Injection		
				Whole Group	Rats with Hb < 12.8 g. per cent.	Rats with Hb > 12.8 g. per cent.
	14	18	18	18	11	7
⁵⁵ Fe (per cent. in spleen)	3.22 ± 1.48	4.69 ± 1.6	1.1 ± 0.692	1.19 ± 0.718	1.47 ± 0.74	0.747 ± 0.424
P		<0.001		>0.7	=0.2	>0.1
⁵⁵ Fe (per cent./g. tissue)	6.5 ± 2.48	7.67 ± 2.57	2.5 ± 1.46	2.37 ± 0.97	2.71 ± 0.97	1.79 ± 0.83
P		=0.2		>0.7	>0.6	>0.1

TABLE VI
⁵⁵Fe IN LIVER 24 HOURS AFTER INTRAPERITONEAL INJECTION OF ⁵⁵Fe IRON-CITRATE
(Results in per cent. of injected doses)

No. of Rats	Controls	After First FA Injection (Whole Group)	Controls	After Second FA Injection		
				Whole Group	Rats with Hb < 12.8 g. per cent.	Rats with Hb > 12.8 g. per cent.
	14	18	18	18	11	7
⁵⁵ Fe (per cent. in liver) ..	10.33 ± 0.2	9.3 ± 1.7	6.83 ± 2.23	4.35 ± 2.35	4.58 ± 2.75	3.91 ± 1.89
P		>0.05		<0.01	>0.04	<0.01
⁵⁵ Fe (per cent./g. tissue)	1.3 ± 0.32	1.34 ± 0.4	0.82 ± 0.164	0.63 ± 0.253	0.657 ± 0.29	0.61 ± 0.18
P		>0.7		>0.001	>0.05	>0.01

Fig. 4.—Incorporation of ^{59}Fe into liver 24 hours after intraperitoneal injection of $^{59}\text{FeHb}$.

Fig. 5.—Incorporation of ^{59}Fe from labelled haemoglobin into spleen 24 hours after intraperitoneal injection of $^{59}\text{FeHb}$.

of ^{59}Fe from ^{59}Fe -citrate than the spleen of intact rats. The uptake of ^{59}Fe from Fe citrate was significantly higher after two injections than in controls, but non-anaemic rats of this group accumulated a smaller amount of radioactive iron than the control rats.

In the same conditions the radioactivity uptake in the liver was lower after FA injection (particularly after the second one) than in the intact rats. The accumulation of ^{59}Fe introduced as labelled haemoglobin into the organs was examined after two injections of FA. The percentage of $^{59}\text{FeHb}$ in the spleen was on the average similar to that in the control group, but anaemic rats had less haemoglobin Fe in the spleens and non-anaemic rats had more than intact animals.

In the liver also a significantly higher amount of Fe from haemoglobin accumulated in non-anaemic rats injected twice with FA. In anaemic rats the radioactivity was nearly equal to that in the control rats.

During examination of the excretion of ^{59}Fe after intraperitoneal injection of radioactive iron citrate,

scarcely measurable traces of ^{59}Fe were found in the urine of intact rats and of rats treated with FA. Therefore only the results of faecal excretion after two injections of FA are presented. At the beginning of the observations the control rats excreted higher amounts of ^{59}Fe , but from the 7th day excretion became low and nearly constant. On the other hand, in the experimental group, a rapid transient increase of excreted ^{59}Fe was observed during the exacerbation of general disease induced by the second injection of FA (Fig. 6).

Fig. 6.—Excretion of ^{59}Fe in rat faeces.

The survival of erythrocytes labelled *in vivo* with ^{51}Cr sodium chromate was examined in intact rats and after a single FA injection. Fig. 7 shows that a moderate but significant shortening of erythrocyte life-span was demonstrated in the rats treated with FA. In this group the half life was 10.15 days compared to 13.4 days in the controls. Complete disappearance of radioactivity from the erythrocytes was seen in 48 ± 4 and 59 ± 2 days respectively.

Discussion

Lukens, Cartwright, and Wintrobe (1967) have shown that, after a single FA injection into the rat, disturbances of iron metabolism occur similar to those observed in the human anaemia of chronic inflammatory states. These disturbances include lowering of the serum iron level and serum iron-binding capacity and increased accumulation of iron in the spleen, liver, and bone marrow reticulum cells. Our examinations were performed during

Fig. 7.—Survival of ⁵¹Cr labelled erythrocytes.

acute attacks of disease after repeated injections of FA.

The serum iron level was found to decrease significantly after each FA dose, even in rats which did not exhibit pronounced anaemia.

The serum iron-binding capacity was unchanged during the exacerbation of general symptoms after two injections of FA. The increased content of non-haeme iron was found only in the spleen and only after the first injection of FA. Further treatment with FA was followed by a lowering of non-haeme iron in the spleen and liver which was quite pronounced during the third FA injection.

The significantly increased accumulation of radioactive iron from ⁵⁹Fe iron citrate was also observed to occur in the spleen only and also only after one injection of FA. The uptake of this form of iron by the liver was low after both single and repeated FA injections. On the other hand the radioactive iron originating from ⁵⁹Fe-labelled haemoglobin was accumulated in great amounts in spleens and livers of rats without anaemia treated repeatedly with FA.

The incorporation of radioactive iron into circulating red blood cells was found to be markedly higher in adjuvant disease than in controls, particularly in the anaemic rats. The augmented utilization of iron by erythrocytes was observed independently of the form of injected radioactive iron, and took place in the case of ⁵⁹Fe iron citrate as well as of ⁵⁹Fe-labelled haemoglobin as the source of incorporated iron.

Two further observations are worth while stressing. They are the shortening of erythrocyte life span already after the first FA injection and increased excretion of iron through the gastrointestinal tract after repeated FA injections. Iron metabolism disturbances in severe anaemia occurring after repeated FA injections differ from those considered as characteristic for the anaemia of chronic inflammatory states (Cartwright, 1966; Lukens and others, 1967; Keiderling and Schmidt, 1959; Hemmeler, 1951; Heilmeyer and Plötner, 1937) in one respect. This is the marked decrease of iron content in spleen and liver after repeated FA injections.

The increased iron excretion through the gastrointestinal tract observed in our experiments which was probably due to internal bleeding may be a partial explanation. Occasionally observed chronic bleeding from inflammatory lesions of the feet may be another mechanism of iron loss. The reasons for these haemorrhagic tendencies are not known but haemostatic disturbances due to vessel damage and clotting changes are not excluded. Nothing is known about the absorption of iron from the gastrointestinal tract in adjuvant disease.

Some of our observations may be considered as consistent with the hypothesis postulated by several authors that in anaemia of inflammation there exists an increased affinity of tissues for iron particularly for iron originating from red cell destruction and as a result there is an impaired utilization and reutilization of iron for haemopoiesis (Freireich, Miller, Emerson, and Ross, 1957; Haurani and Burke, 1964; Haurani, Burke, and Martinez, 1965; Quastel and Ross, 1966). The increased content of iron in the spleen after one FA injection may be explained by this mechanism although shortened erythrocyte survival may be another contributing factor.

The augmented accumulation of ⁵⁹Fe from labelled haemoglobin in spleen and liver after repeated injections of FA in the rats without pronounced anaemia is also consistent with this hypothesis. The increased incorporation of radioactive iron from ⁵⁹FeHb into circulating erythrocytes in the rats with pronounced anaemia after repeated FA injections cannot exclude the possibility of the existence of impaired utilization of iron originating from intracellular haemolysis.

There are perhaps some differences between the fate of iron in intraperitoneally introduced haemoglobin and that of iron from the red cells destroyed in the reticulo-endothelial system.

In this group of rats with pronounced anaemia increased haemolysis and blood losses seem to be

additional pathogenic mechanisms beside disturbances in iron metabolism. The enhanced stimulation of erythropoietic activity may explain the observed high incorporation of ^{59}Fe both from citrate and labelled haemoglobin.

Summarizing, we were able to demonstrate that FA injections induce deep changes in iron metabolism, shorten the erythrocyte survival time, and increase iron excretion through the gastrointestinal tract. This last effect is of importance in repeated attacks of adjuvant disease and is a probable reason for the similarity of anaemia in these rats to anaemia from true iron deficiency.

Summary

Iron metabolism and erythrocyte survival were studied in rats injected intracutaneously into the

foot pad with FA. The level of iron in the serum decreased significantly. Incorporation of ^{59}Fe (given as Fe-citrate or in haemoglobin) increased in the experimental animals, particularly in rats with anaemia.

Non-haeme iron and ^{59}Fe increased in the spleen of animals treated once with FA. These values decreased, however, in rats which received repeated injections. Non-haeme iron in the liver decreased. In the liver and spleen of experimental animals without anaemia there was an increase in $^{59}\text{FeHb}$ accumulation.

In the experimental animals there was an increase in ^{59}Fe excretion through the gastrointestinal tract and a shortening of erythrocyte survival time.

REFERENCES

- Cartwright, G. E. (1966). *Semin. Hemat.*, **3**, 351 (The anemia of chronic disorders).
- Drabkin, D. L. (1950). *J. biol. Chem.*, **185**, 231 (Spectrophotometric studies. XV. Hydration of macro-sized crystals of human hemoglobin, and osmotic concentrations in red cells).
- Freireich, E. J., Miller, A., Emerson, P., and Ross, J. F. (1957). *Blood*, **12**, 972 (The effect of inflammation on the utilization of erythrocyte and transferrin bound radioiron for red cell production).
- Haurani, F. I., and Burke, W. (1964). "X Congress of the International Society of Haematology", F-38 Stockholm (Iron reutilization in the anaemia of inflammation).
- , —, and Martinez, E. J. (1965). *J. Lab. clin. Med.*, **65**, 560 (Defective reutilization of iron in the anemia of inflammation).
- Heilmeyer, L., and Plötner, K. (1937). "Das Serumeisen und die Eisenmangelkrankheit", Fischer, Jena.
- Hemmeler, G. (1951). "Métabolisme du fer". Masson, Paris.
- Keiderling, W., and Schmidt, H. A. E. (1959). In "Eisenstoffwechsel", ed. W. Keiderling, p. 155. Thieme, Stuttgart.
- Lukens, J. N., Cartwright, G. E., and Wintrobe, M. M. (1967). *Proc. Soc. exp. Biol. (N.Y.)*, **126**, 346 (Anemia of adjuvant-induced inflammation in rats).
- Mikolajew, M., Kuratowska, Z., Kossakowska, M., Plachecka, M., and Kopeć, M. (1969). *Ann. rheum. Dis.*, **28**, 35 (Haematological changes in adjuvant disease in the rat. I. Peripheral blood and bone marrow after repeated injections of Freund adjuvant).
- Quastel, M. R., and Ross, J. F. (1966). *Blood*, **28**, 738 (The effect of acute inflammation on the utilization and distribution of transferrin-bound and erythrocyte radioiron).
- Ramsay, W. N. M. (1957). *Clin. chim. Acta*, **2**, 221 (The determination of the total iron-binding capacity of serum).
- Schales, O. (1958). "Iron in Serum", in "Standard Methods of Clinical Chemistry", ed. D. Seligson, vol. 2, p. 69. Academic Press, New York.

Les changements hématologiques dans la maladie causée par un adjuvant chez le rat. II. Le métabolisme du fer et la survivance de l'érythrocyte ^{51}Cr .

RÉSUMÉ

Le métabolisme du fer et la survivance de l'érythrocyte ont été étudiés chez les rats injectés par voie intradermique dans la pulpe du pied avec l'adjuvant de Freund (AF). Le taux du fer dans le sérum avait diminué d'une façon marquée. L'incorporation du ^{59}Fe (donné comme citrate de fer ou dans l'hémoglobine) augmentait dans les animaux prenant part à l'expérience, particulièrement chez les rats atteints d'anémie.

Le fer non-hémo et le ^{59}Fe augmentaient dans la rate des animaux qui avaient reçu le AF une seule fois. Ces valeurs diminuaient, cependant, chez les rats qui avaient reçu des injections répétées. Le fer non-hémo dans le foie avait diminué. Dans le foie et la rate des animaux sans anémie compris dans l'expérience, il y avait une augmentation dans l'accumulation du $^{59}\text{FeHb}$.

Chez les animaux de l'expérience il y avait une augmentation dans l'excrétion du ^{59}Fe par la voie gastro-intestinale et une diminution de la période de survivance de l'érythrocyte.

Cambios hematológicos en enfermedad adyuvante en la rata. II. Metabolismo del hierro y ^{51}Cr sobrevivencia de eritrocitos

SUMARIO

El metabolismo del hierro y la sobrevivencia de eritrocitos fueron estudiados en ratas inyectadas intracutáneamente en los extremos de las patas con adyuvante de Freund (FA). El nivel de hierro en el suero disminuyó significativamente. La incorporación de ^{59}Fe (administrado en forma de Fe-citrato o en hemoglobina) aumentó en los animales del experimento, particularmente en ratas con anemia.

El hierro no hemoglobínico y el ^{59}Fe aumentaron en el bazo de los animales tratados una vez con FA. Estos valores disminuyeron, sin embargo, en ratas que habían recibido varias inyecciones. El hierro no hemoglobínico disminuyó en el hígado. En el hígado y el bazo de animales de experimento, sin anemia, hubo un incremento en la acumulación de $^{59}\text{FeHb}$. En los animales del experimento hubo un aumento en la excreción de ^{59}Fe a través del tracto gastrointestinal y una reducción en el período de sobrevivencia de eritrocitos.